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(54) Title: INSULIN CRYSTALS FOR PULMONARY ADMINISTRATION

(57) Abstract: The invention relates to crystals comprising insulin, an insulin analog, derivatives of insulin or derivatives of an insulin analog, and a divalent metal cation, to processes for preparing such crystals, and to methods of treating diabetes or hyperglycemia comprising administering the crystals via the pulmonary route to a patient in need thereof to control blood glucose. Crystals having a volume mean spherical equivalent diameter of 1 to 5 microns are obtained. The crystal size may be controlled so that the crystals may be administered advantageously by inhalation through the mouth, deposited in the deep lung, and absorbed there over a protracted period of time, to provide control of blood glucose primarily between meals and overnight.

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INSULIN CRYSTALS FOR PULMONARY ADMINISTRATION

Cross Reference

5 This application claims the benefit of U.S. Provisional Application No. 60/141,430 filed on June 29, 1999, said application of which is entirely incorporated herein by reference

10 **Background of the Invention**

15 1. Field of the Invention. The invention is in the field of human medicine. More particularly, the invention is in the field of the treatment of diabetes and hyperglycemia.

20 2. Description of Related Art. It has long been a goal of insulin therapy to mimic the pattern of endogenous insulin secretion in normal individuals. The daily physiological demand for insulin fluctuates and can be separated into two phases: (a) the absorptive phase requiring a pulse of insulin to dispose of the meal-related blood glucose surge, and (b) the post-absorptive phase requiring a sustained delivery of insulin to regulate hepatic glucose output for maintaining optimal fasting blood glucose. Accordingly, effective therapy for people with diabetes generally involves the combined use of two types of exogenous insulin formulations: a fast-acting meal time insulin provided by bolus injections and a long-acting, so-called, basal insulin, administered by injection once or twice daily to control blood glucose levels between meals.

25 Conventional insulin therapy involves only two injections per day. More intensive insulin therapy involving three or more injections of insulin each day

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results in reduction of complications, as demonstrated in the Diabetes Control and Complications Trial (DCCT) study. Unfortunately, many diabetics are unwilling to undertake intensive therapy due to the discomfort associated with the 5 many injections required to maintain close control of glucose levels. A non-injectable form of insulin is desirable for increasing patient compliance with intensive insulin therapy and lowering their risk of complications.

Many investigators have studied non-injectable 10 forms of insulin for delivery by oral, rectal, transdermal, and nasal routes. So far, these types of administration have not been effective due to poor insulin absorption, low serum insulin concentration, irritation at the site of delivery, or lack of significant decrease in serum glucose 15 levels.

Another well-studied route for administering non-injectable insulin is by the lung. Due to its relatively small molecular weight (5,800 daltons) insulin seems to be an ideal candidate for administration through the lungs. 20 Administration of insulin by inhalation, and its absorption through the lung was first reported in 1925.

However, after administration by inhalation, small-sized proteins like insulin are absorbed rapidly from the lung due to the very large surface area and relatively 25 porous membrane of the lung. The plasma concentration peaks quickly, but also decreases quickly. These pharmacokinetics may be suited for controlling blood glucose during the absorptive phase, but are completely unsuited during the post-absorptive phase. Therefore, means for administration 30 of long-acting insulin by inhalation remains a challenge.

The following are reviews of the inhalation of insulin and other proteins: "Aerosol Insulin- A Brief Review", Patton, J. S. and Platz, R. M., in *Respiratory Drug*

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- Delivery IV*, P. Byron, Ed., Interpharm Press (1994); "Delivery of Biotherapeutics by Inhalation Aerosol", Niven, R., *Crit. Rev. in Therapeutic Drug Carrier Systems* 12:151-231 (1995); and "Drug Delivery via the Respiratory Tract", 5 Byron, P. R. and Patton, J. S., *J. Aerosol Medicine* 7:49-75 (1994).

The efficiency of delivery and deposition of particles onto the surface of the deep lung, i.e. to the alveoli, is strongly related to the volume mean spherical 10 equivalent diameter (VMSED) of the particles. The optimal volume mean spherical equivalent diameter is about 2-3 microns. Above and below this range, less material will be deposited onto the alveolar surface.

Phagocytosis of insoluble slowly dissolving 15 particles is the most significant clearance mechanism in the deep lung [Rudt, S. H., et al., *J. Controlled Release* 25:123-138 (1993); Tabata, Y., et al., *J. Biomedical Material Res.* 22:837-842 (1988); Wang, J. A., et al., In: *Respiratory Drug Delivery*. Buffalo Grove, IL; Interpharm 20 1997, vol. VI, pp. 187-192; Edwards, D. A., et al., *Science* 276:1868-1871 (1997); Gehr, P. M., *Microsc. Res. Tech.* 26:423-436 (1993)]. Once deposited on the alveolar surface, particles having actual dimensions in the range of about 1 to about 10 microns may be phagocytosed by lung macrophages. 25 It is known that very large particles, such as those having actual diameters above about 10 microns, are not efficiently phagocytosed by lung macrophages. Small particles with actual dimensions in the nanometer size range, likely also escape macrophage ingestion.

30 Unfortunately, particles having optimal properties for delivery and deposition often have actual particle dimensions that fall in the range within which macrophage attack is expected.

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Edwards, et al. describe protracted release of insulin from large, porous particles administered into the deep lung by inhalation [J. Appl. Physiol. 85:379-385 (1998); Science 276:1868-1871 (1997)]. The persistence of 5 the administered particles was attributed to the large actual size of the particles (greater than 10 microns), while their high rate of deposition into the deep lung was attributed to the low density of the particles which resulted in relatively small aerodynamic size despite their 10 large physical size. These particles comprise insulin encapsulated in a biodegradable copolymer (poly-lactic-co-glycolic acid). Slow degradation of the copolymer releases insulin over a period of at least four days. These publications demonstrate that a depot effect in the lung is 15 a feasible mechanism for producing a sustained delivery of insulin.

Crystallization of insulin, such as with NPH-insulin and the various Lente products, is a well-known means to provide extended control of blood glucose in people 20 with diabetes. Insulin crystals have been administered by parenteral routes, usually subcutaneously. Sufficiently long duration of insulin-containing crystals when delivered to the deep lung by inhalation has not been achieved. This is likely due to the complex protective mechanisms involving 25 the biology, immunology, and chemistry of the lung surface relating to clearing of air-borne particles, especially microbes and aerosols containing microbes, which have particle qualities that permit their evasion of upper respiratory entrapment mechanisms.

30 Hughes, B. L., et al. [PCT/US98/23040, filed 29 October 1998; EP 0 919 242 A2, published 02 June 1999] described pharmaceutical compositions and methods of administering fatty acid-acylated insulin and insulin

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analogs by inhalation to treat diabetes. The compositions were solutions or powders of amorphous materials, but not crystals. Hughes, et al. demonstrated that derivatized insulins, B28-Nε-myristoyl-LysB28, ProB29-human insulin analog and B29-Nε-palmitoyl-human insulin, were absorbed in an amount effective to reduce glucose levels. The pharmacokinetics of the fatty acid-acylated insulins administered via the lung was protracted compared with non-acylated insulin, but the length of protraction was shorter than that of NPH-insulin delivered subcutaneously.

Havelund, S. [WO98/42749, published 1 October 1998] describes zinc-free crystal powders of insulin, insulin analogs, and insulin derivatives that are claimed to be resistant to aggregation and clumping, and allegedly suitable for administration by inhalation.

Whittingham, J. L., et al. [Biochemistry 36:2826-2831 (1997)] produced very large crystals comprised of B29-Nε-tetradecanoyl-des(B30)-human insulin analog and zinc for structural studies by X-ray crystallography. These crystals were much too large to expect efficient deposition in the deep lung when administered by inhalation. These crystals would have to be milled to produce material of suitable particle size to achieve efficient deposition when administered by inhalation.

Brader, M. [U.S. Patent Application No. 09/177685, filed 22 October 1998; PCT/US98/22434; European Patent Publication No. 0911035, published 28 April 1999, herein Brader I] and Brader, M. [U.S. Patent Application No. 09/217275, filed 21 December 1998; PCT/US98/27299, herein Brader II] describe microcrystals comprising divalent metal cations together with derivatized proteins, including derivatized insulin and derivatized insulin analogs,

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processes for making the crystals, and methods for administering them to treat diabetes. The crystals are rod-shaped, and have the size of rod-shaped commercial NPH-insulin crystals, which is about 5 microns in length. Such 5 crystals are thought to be too large to obtain optimal deposition in the deep lung when administered by inhalation through the mouth.

Another disadvantage to the NPH-type crystals of Brader I and Brader II are their incorporation of the fish 10 protein, protamine. While it was earlier believed that protamines were non-immunogenic, it is now believed that protamines can be immunogenic in man and that their use for medical purposes may lead to formation of antibodies [Samuel, et.al., Clin. Exp. Immunol. 33, pp. 252-260 15 (1978)].

Also, evidence has been found that the protamine-insulin complex is itself immunogenic [Kurtz, et.al., Diabetologia 25, pp. 322-324, (1983)]. Therefore, with some 20 patients the use of protracted insulin compositions containing protamines must be avoided.

It has been known for many years that insulin can be successfully combined with zinc ions to obtain numerous types of stable crystals with longer time actions than soluble or amorphous, uncristallized insulin. In the early 25 1950s, a new formulation of beef insulin crystals was developed which contained only insulin and zinc in an acetate buffer at neutral pH [Hallas-Møller, et.al., Science 116, 394-398 (1952)]. This insulin preparation avoided phosphate ions, which interact strongly with zinc ions to 30 form insoluble zinc phosphate derivatives. Formulations containing only crystalline insulin in acetate buffer are called Ultralente. Crystals prepared in this manner will be referred herein as Ultralente-type crystals. Most

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significantly, Ultralente-type crystals avoid the use of protamines. Other references to the production of Ultralente-type crystals are Schlichtkrull, et.al., U.S. Patent 2,799,622 issued July 16, 1957 (referred to 5 hereinafter as Schlichtkrull I) Schlichtkrull, et.al., U.S. Patent 2,819,999 issued Jan. 14, 1958 (referred to hereinafter as Schlichtkrull II), Hoffmann, U.S. Patent 10 5,534,488 issued July 9, 1996 and in publications such as Galenics of Insulin, by Brange, Springer-Verlag, New York 10 (1987) and Insulin Crystals, by Schlichtkrull, Ejnar Munksgaard Publishers, Copenhagen (1958), referred to hereinafter as Schlichtkrull III.

Schlichtkrull II describes processes for the production of seed crystals of animal insulins of 15 substantially the same size within the range of about 1 micron to about 7 microns. The crystals are preferably used in a "nucleating seed suspension" for the production of larger insulin crystals of uniform size.

Schlichtkrull I describes the use of these animal 20 seed crystals in a "nucleating seed suspension" for the manufacture of animal insulin crystals of substantially uniform size within the range of 10 microns to about 40 microns. Schlichtkrull I notes that it is not appropriate to use crystals smaller than about 10 microns in size 25 because these very small insulin crystals result in a decreased protracted effect.

A commercial product derived from this insulin crystallization is beef Ultralente. This insulin product, injected subcutaneously, provides a slow, steady infusion of 30 insulin into the bloodstream that matches the low level of glucose output from the liver without any noticeable insulin peak (Owens, et.al., Diabetic Medicine 3, 326-329 (1986)).

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A major problem with beef Ultralente, however, stems from the fact that beef insulin is different in amino acid sequence from human insulin. The human body can recognize bovine insulin as a foreign protein. Chronic administration of this immunogenic substance in diabetic patients can result in formation of antibodies to the insulin. This can lead to alterations in insulin time action and potency and other problems arising from the patient's activated immune system.

The advent of recombinant DNA technology and novel enzymatic techniques for converting pork insulin into human insulin both resulted in abundant supplies of human insulin becoming available in 1980. To overcome the problems associated with beef Ultralente noted above, a logical step was the preparation of human Ultralente crystals and their formulation into a commercially available, parenteral formulation. The crystal forms, crystal shapes, crystal sizes, method of preparation and formulation compositions of human and beef Ultralente products are essentially identical.

However, several years of clinical experience led to definite indications that these products were not identical. In fact, clinical reports indicated that human Ultralente was faster acting than the comparable beef Ultralente formulation. In clinical practice, this has led many physicians and diabetologists to recommend a twice a day injection protocol for human Ultralente. In addition, a significant peak of insulin absorption into the blood stream is observed about 12 hours after subcutaneous administration.

It is well known in the art that Ultralente-type insulin crystals in pharmaceutical formulations are rhombohedral crystals with an average diameter of about 25

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microns. Hallas-Møller et. al. also notes that subcutaneously injected preparations of larger-sized crystals have a more protracted biological time action, while smaller crystals obtained by grinding have a somewhat 5 faster time action. Grinding or milling operations themselves are inconvenient steps for the production of insulin crystals of the desired particle size distribution.

Surprisingly, the present invention provides crystals and pharmaceutical compositions of insulins that 10 avoid incorporation of protamine, avoid incorporation of beef insulin, do not entail milling or grinding in their preparation, have a VMSED of 1 to 5 microns with a uni-modal particle size distribution that are suitable for pulmonary administration and yet retain an extended time action of 15 biological activity after administration.

Summary of the Invention

The invention includes a substantially uniform population of Ultralente-type crystals comprised of insulin, 20 an insulin analog, a derivatized insulin, or insulin analog and a divalent metal cation, characterized in that the crystals have a volume mean spherical equivalent diameter (VMSED) particle size in the range of 1 to 5 microns.

More specifically, the invention is Ultralente-type crystals having a uni-modal, particle size 25 distribution, comprising:

- a) insulin, an insulin analog or a derivatized insulin or insulin analog, and
 - b) a divalent metal cation;
- 30 characterized in that the volume mean spherical equivalent diameter is from 1 microns to 5 microns.

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The invention also includes pharmaceutical compositions comprising crystals together with one or more pharmaceutically acceptable excipient carriers, or with an aqueous solvent in which the crystals are stable. The 5 pharmaceutical composition is suitable and useful for administration by inhalation through the mouth of the patient for deposition in the deep lung (the alveolae).

For pharmaceutical compositions for pulmonary administration, the crystals may be in the form of a dry 10 powder (for delivery by a dry powder inhaler for example), optionally together with other dry excipients, dry powders of amorphous insulin or an insulin analog, and carrier particles. The crystals may also be formulated for administration by inhalation through the mouth of the 15 patient in a liquid form by suspending them in a pharmaceutically acceptable aqueous solvent, optionally together with pharmaceutically acceptable buffers, excipients and preservatives.

The invention further provides processes for 20 preparing crystals of a size that increases the efficiency of their deposition in the deep lung when administered by inhalation through the mouth. The process involves preparation of a herein-defined "protein solution", generally at acidic pH, comprising insulin, an insulin 25 analog or a derivatized insulin or insulin analog. Also prepared is a herein-defined "precipitation solution", generally at an alkaline pH, comprising salts such as sodium chloride sodium acetate and optionally other components. After combination of these two solutions to form a herein- 30 defined "crystallization solution", a "nucleating seed suspension" is added comprising preferably microcrystals of insulin and commonly used in the manufacture of Ultralente-type insulin products. The Ultralente-like crystals of the

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present invention form spontaneously, preferably at ambient temperature with mechanical stirring. The crystallization process is preferably complete in about 3 to about 25 hours.

The invention also encompasses the use of crystals
5 in the manufacture of a medicament for the treatment of diabetes or hyperglycemia by inhalation, which treatment comprises administering to a patient in need thereof an effective amount of the medicament using an inhalation device, such that the medicament is deposited in the lungs
10 of the patient.

The present invention also provides a method for administering the crystals by inhalation.

The present invention solves several problems currently not addressed by the art. First, this invention
15 provides methods for delivering insulin by pulmonary means that provide adequate time action to control blood glucose between meals and overnight. Second, this invention provides methods for delivering insulin by pulmonary means which avoids the subcutaneous injections of presently known
20 soluble or insoluble, long-acting insulins which involves the inconvenience of preparing a sample for injection, and the pain of a needle-stick. Thirdly, the potentially immunogenic protein protamine presently incorporated into NPH-like crystals and pharmaceutical compositions is
25 avoided. Fourthly, grinding, milling or other harsh or inconvenient post-crystallization steps normally required to reduce commercially available insulin crystals to the appropriate diameter for pulmonary delivery are avoided.

According to the present invention, a patient in
30 need of insulin to control blood glucose levels will benefit from an advantageous slow uptake and prolonged persistence of insulin activity and also a reduction of inconvenience and pain compared with subcutaneous delivery. The crystals

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can be delivered in a carrier, as a suspension or as a dry powder, using any of a variety of devices suitable for administration by inhalation. The insulin can be administered using an inhalation device such as a nebulizer,
5 a metered-dose inhaler, a dry powder inhaler, a sprayer, and the like.

The invention also provides a method for administering a pharmaceutical composition comprising either crystals of insulin, an insulin analog, or derivatives thereof to a patient in need thereof by inhalation.
10 Administering such crystals provides basal control of blood glucose levels. In the preferred dry powder administration, faster acting amorphous preparations of insulin, insulin analog, animal insulin other than beef insulin or
15 derivatives thereof may be added to the crystals to provide post-prandial as well as basal control of blood glucose levels. Other excipients such as zinc may be added to the dry powder to further prolong the time action of the crystals. Because the method avoids injections, patient
20 comfort is improved and patient compliance is increased compared with conventional insulin delivery methods.

Detailed Description of the Invention

25 As used herein, the term "crystal" means a microcrystal comprising insulin, an insulin analog, a derivatized insulin, or derivatized insulin analog.

30 The term "microcrystal" means a solid that is comprised primarily of matter in a crystalline state, and are of a microscopic size, typically of longest dimension within the range 1 micron to 10 microns. The term "microcrystalline" refers to the state of being a microcrystal.

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The term "protein" may have its common meaning, that is, a polymer of amino acids. The term "protein," as used herein, also has a narrower meaning, that is, a protein selected from the group consisting of insulin or insulin analogs.

The term "derivatized protein" refers to a protein selected from the group consisting of derivatized insulin, derivatized insulin analogs and derivatized animal insulins that is derivatized by a functional group such that the derivatized protein is either less soluble in an aqueous solvent than is the un-derivatized protein or is more lipophilic than un-derivatized insulin. The determination of either the solubility or lipophilicity of proteins and derivatized proteins is well-known to the skilled person.

The solubility of derivatized proteins and protein in complexes with zinc can be readily determined by well-known procedures [Graham and Pomeroy, *J. Pharm. Pharmacol.* 36:427-430 (1983), as modified in DeFelippis, M. R. and Frank, B., EP 735,048].

Many examples of such derivatized proteins are known in the art, including benzoyl, *p*-tolyl-sulfonamide carbonyl, and indolyl derivatives of insulin and insulin analogs [Havelund, S., et al., WO95/07931, published 23 March 1995]; alkylloxycarbonyl derivatives of insulin [Geiger, R., et al., U.S. Patent No. 3,684,791, issued 15 August 1972; Brandenberg, D., et al., U.S. 3,907,763, issued 23 September 1975]; aryloxycarbonyl derivatives of insulin [Brandenberg, D., et al., U.S. 3,907,763, issued 23 September 1975]; alkylcarbamyl derivatives [Smyth, D. G., U.S. Patent No. 3,864,325, issued 4 February 1975; Lindsay, D. G., et al., U.S. Patent No. 3,950,517, issued 13 April 1976]; carbamyl, O-acetyl derivatives of insulin [Smyth, D. G., U.S. Patent No. 3,864,325 issued 4 February 1975];

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cross-linked, alkyl dicarboxyl derivatives [Brandenberg, D., et al., U.S. Patent No. 3,907,763, issued 23 September 1975]; N-carbamyl, O-acetylated insulin derivatives [Smyth, D. G., U.S. Patent No. 3,868,356, issued 25 February 1975];
5 various O-alkyl esters [Markussen, J., U.S. Patent No. 4,343,898, issued 10 August 1982; Morihara, K., et al., U.S. Patent No. 4,400,465, issued 23 August 1983; Morihara, K., et al., U.S. Patent No. 4,401,757, issued 30 August 1983; Markussen, J., U.S. Patent No. 4,489,159, issued 18 December
10 1984; Obermeier, R., et al., U.S. Patent No. 4,601,852, issued 22 July 1986; and Andersen, F. H., et al., U.S. Patent No. 4,601,979, issued 22 July 1986]; alkylamide derivatives of insulin [Balschmidt, P., et al., U.S. Patent No. 5,430,016, issued 4 July 1995]; various other
15 derivatives of insulin [Lindsay, D. G., U.S. Patent No. 3,869,437, issued 4 March 1975]; and the fatty acid-acylated proteins that are described herein.

The term "acylated protein" as used herein refers to a derivatized protein selected from the group consisting of insulin and insulin analogs that is acylated with an organic acid moiety that is bonded to the protein through an amide bond formed between the acid group of an organic acid compound and an amino group of the protein. In general, the amino group may be the α -amino group of an N-terminal amino acid of the protein, or may be the ϵ -amino group of a Lys residue of the protein. An acylated protein may be acylated at one or more of the three amino groups that are present in insulin and in most insulin analogs. Mono-acylated proteins are acylated at a single amino group. Di-acylated proteins are acylated at two amino groups. Tri-acylated proteins are acylated at three amino groups. The organic acid compound may be, for example, a fatty acid, an aromatic acid, or any other organic compound having a carboxylic acid group that

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will form an amide bond with an amino group of a protein, and that will lower the aqueous solubility, raise the lipophilicity, or decrease the solubility of zinc complexes of the derivatized protein compared with the un-derivatized 5 protein.

The term "fatty acid-acylated protein" refers to a an acylated protein selected from the group consisting of insulin and insulin analogs that is acylated with a fatty acid that is bonded to the protein through an amide bond 10 formed between the acid group of the fatty acid and an amino group of the protein. In general, the amino group may be the α -amino group of an N-terminal amino acid of the protein, or may be the ϵ -amino group of a Lys residue of the protein. A fatty acid-acylated protein may be acylated at 15 one or more of the three amino groups that are present in insulin and in most insulin analogs. Mono-acylated proteins are acylated at a single amino group. Di-acylated proteins are acylated at two amino groups. Tri-acylated proteins are acylated at three amino groups. Fatty acid-acylated insulin 20 is disclosed in a Japanese patent application 1-254,699. See also, Hashimoto, M., et al., *Pharmaceutical Research*, 6:171-176 (1989), and Lindsay, D. G., et al., *Biochemical J.* 121:737-745 (1971). Further disclosure of fatty acid-acylated insulins and fatty acylated insulin analogs, and of 25 methods for their synthesis, is found in Baker, J. C., et al, U.S. 08/342,931, filed 17 November 1994 and issued as U.S. Patent No. 5,693,609, 2 December 1997; Havelund, S., et al., WO95/07931, published 23 March 1995, and a corresponding U.S. Patent No. 5,750,497, 12 May 1998; and 30 Jonassen, I., et al., WO96/29342, published 26 September 1996.

The term "fatty acid-acylated protein" includes pharmaceutically acceptable salts and complexes of fatty

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acid-acylated proteins. The term "fatty acid-acylated protein" also includes preparations of acylated proteins wherein the population of acylated protein molecules is homogeneous with respect to the site of acylation. For example, N ϵ -mono-acylated protein, B1-N α -mono-acylated protein, A1-N α -mono-acylated protein, A1,B1-N α -di-acylated protein, N ϵ ,A1-N α ,di-acylated protein, N ϵ ,B1-N α ,di-acylated protein, and N ϵ ,A1,B1-N α ,tri-acylated protein are all encompassed within the term "fatty acid-acylated protein" for the purpose of the present invention. The term also refers to preparations wherein the population of acylated protein molecules has heterogeneous acylation. In the latter case, the term "fatty acid-acylated protein" includes mixtures of mono-acylated and di-acylated proteins, mixtures of mono-acylated and tri-acylated proteins, mixtures of di-acylated and tri-acylated proteins, and mixtures of mono-acylated, di-acylated, and tri-acylated proteins.

The term "pI" stands for "isoelectric point". Methods for calculating or experimentally determining the isoelectric point of insulin-related proteins and other molecules are known to one of ordinary skill in the art.

The term "insulin" as used herein, refers to human insulin, whose amino acid sequence structure is well-known. Human insulin is comprised of a twenty-one amino acid A-chain and a thirty-amino acid B-chain which are cross-linked by disulfide bonds. A properly cross-linked insulin contains three disulfide bridges: one between position 7 of the A-chain and position 7 of the B-chain, a second between position 20 of the A-chain and position 19 of the B-chain, and a third between positions 6 and 11 of the A-chain.

The term "insulin analog" as used herein means proteins that have an A-chain and a B-chain that have

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- substantially the same amino acid sequences as the A-chain and B-chain of human insulin, respectively, but differ from the A-chain and B-chain of human insulin by having one or more amino acid deletions, one or more amino acid
5 replacements, and/or one or more amino acid additions that do not destroy the insulin activity of the insulin analog. Deletions, replacements and additions that combine to form the structures of naturally occurring non-human animal insulins are not included in this definition.
10 "Animal insulins" as used herein refer to the structures of insulin from non-human animals. Four such animal insulins are rabbit, pork, beef, sheep insulin. The amino acid substitutions that distinguish these animal insulins from human insulin are presented below for the
15 reader's convenience.

| | Amino Acid Position | | | |
|----------------|---------------------|-----------|------------|------------|
| | <u>A8</u> | <u>A9</u> | <u>A10</u> | <u>B30</u> |
| human insulin | Thr | Ser | Ile | Thr |
| rabbit insulin | Thr | Ser | Ile | Ser |
| pork insulin | Thr | Ser | Ile | Ala |
| beef insulin | Ala | Ser | Val | Ala |
| sheep insulin | Ala | Gly | Val | Ala |

Another type of insulin analog, "monomeric insulin analog" is well-known in the art. Monomeric insulin analogs are structurally very similar to human insulin, and have
20 activity similar or equal to human insulin, but have one or more amino acid deletions, replacements or additions that tend to disrupt the contacts involved in dimerization and hexamerization which results in their having less tendency to associate to higher aggregation states. Monomeric
25 insulin analogs are rapid-acting analogs of human insulin, and are disclosed, for example, in Chance, R. E., et al.,

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U.S. patent No. 5,514,646, 7 May 1996; Brems, D. N., et al. *Protein Engineering*, 5:527-533 (1992); Brange, J. J. V., et al., EPO publication No. 214,826, published 18 March 1987; Brange, J. J. V., et al., U.S. Patent No. 5,618,913, 8 April 5 1997; and Brange, J., et al., *Current Opinion in Structural Biology* 1:934-940 (1991). An example of monomeric insulin analogs is described as human insulin wherein Pro at position B28 is substituted with Asp, Lys, Leu, Val, or Ala, and wherein Lys at position B29 is Lys or is substituted 10 with Pro, and also, AlaB26-human insulin, des(B28-B30)-human insulin, and des(B27)-human insulin. The monomeric insulin analogs employed as derivatives in the present crystals, or employed un-derivatized in the solution phase of suspension formulations, are properly cross-linked at the same 15 positions as is human insulin.

Another group of insulin analogs for use in the present invention are those wherein the isoelectric point of the insulin analog is between about 7.0 and about 8.0. These analogs are referred to as "pI-shifted insulin 20 analogs." Examples of such insulin analogs include ArgB31,ArgB32-human insulin, GlyA21,ArgB31,ArgB32-human insulin, ArgA0,ArgB31,ArgB32-human insulin, and, ArgA0,GlyA21,ArgB31,ArgB32-human insulin.

Another group of insulin analogs consists of 25 insulin analogs that have one or more amino acid deletions that do not significantly disrupt the activity of the molecule. This group of insulin analogs is designated herein as "deletion analogs." For example, insulin analogs with deletion of one or more amino acids at positions B1-B3 30 are active. Likewise, insulin analogs with deletion of one or more amino acids at positions B28-B30 are active. Examples of "deletion analogs" include des(B30)-human insulin, desPhe(B1)-human insulin, des(B27)-human insulin,

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des(B28-B30)-human insulin, and des(B1-B3)-human insulin. The deletion analogs employed as derivatives in the present crystals, or employed un-derivatized in the solution phase of suspension formulations, are properly cross-linked at the 5 same positions as is human insulin.

Amidated amino acids, and particularly asparagine residues in insulin, are known to be chemically unstable [Jorgensen, K. H., et al. U.S. Patent No. 5,008,241, issued 16 April, 1991; Dorschug, M., U.S. Patent No. 5,656,722, 10 issued 12 August, 1997]. Particularly, they are prone to deamidation and various rearrangement reactions under certain conditions that are well-known. Therefore, optionally, an insulin analog may be insulin or an insulin analog that has one or more of its amidated residues 15 replaced with other amino acids for the sake of chemical stability. For example, Asn or Gln may be replaced with a non-amidated amino acid. Preferred amino acid replacements for Asn or Gln are Gly, Ser, Thr, Asp or Glu. It is preferred to replace one or more Asn residues. In 20 particular, AsnA18, AsnA21, or AsnB3, or any combination of those residues may be replaced by Gly, Asp, or Glu, for example. Also, GlnA15 or GlnB4, or both, may be replaced by either Asp or Glu. Preferred replacements are Asp at B21, and Asp at B3. Also preferred are replacements that do not 25 change the charge on the protein molecule, so that replacement of Asn or Gln with neutral amino acids is also preferred.

A "pharmaceutically acceptable salt" means a salt formed between any one or more of the charged groups in a 30 protein and any one or more pharmaceutically acceptable, non-toxic cations or anions. Organic and inorganic salts include, for example, those prepared from acids such as hydrochloric, sulfuric, sulfonic, tartaric, fumaric,

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hydrobromic, glycolic, citric, maleic, phosphoric, succinic, acetic, nitric, benzoic, ascorbic, p-toluenesulfonic, benzenesulfonic, naphthalenesulfonic, propionic, carbonic, and the like, or for example, ammonium, sodium, potassium, 5 calcium, or magnesium.

The verb "acylate" means to form the amide bond between a fatty acid and an amino group of a protein. A protein is "acylated" when one or more of its amino groups is combined in an amide bond with the acid group of a fatty 10 acid.

The term "fatty acid" means a saturated or unsaturated, straight chain or branched chain fatty acid, having from one to eighteen carbon atoms.

The term "C1 to C18 fatty acid" refers to a 15 saturated, straight chain or branched chain fatty acid having from one to eighteen carbon atoms.

The term "divalent metal cation" refers to the ion or ions that participate to form a complex with a multiplicity of protein molecules. The transition metals, 20 the alkaline metals, and the alkaline earth metals are examples of metals that are known to form complexes with insulin. The transitional metals are preferred. Zinc is particularly preferred. Other transition metals that may be pharmaceutically acceptable for complexing with insulin 25 proteins include copper, cobalt, and iron.

The term "zinc ion" is understood to one skilled in the art. The source of the zinc ion is preferably a zinc salt. Representative examples of zinc salts included zinc acetate, zinc bromide, zinc chloride, zinc fluoride, zinc 30 iodide and zinc sulfate. The skilled artisan will recognize that there are many other zinc salts which also might be used in the production of the formulations of the present invention.

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The term "complex" refers to a complex formed between one or more atoms in the proteins that form the complex and one or more divalent metal cations. The atoms in the proteins serve as electron-donating ligands. The 5 insulin proteins typically form a hexamer complex with divalent transition metal

The term "suspension" refers to a mixture of a liquid phase and a solid phase that consists of insoluble sparingly soluble particles that are larger than colloidal 10 size. Mixtures of Ultralente-type crystals and an aqueous solvent form suspensions. The term "suspension formulation" means a pharmaceutical composition wherein an active agent is present in a solid phase, for example, a microcrystalline solid, an amorphous precipitate, or both, which is finely 15 dispersed in an aqueous solvent. The finely dispersed solid is such that it may be suspended in a fairly uniform manner throughout the aqueous solvent by the action of gently agitating the mixture, thus providing a reasonably uniform suspension from which a dosage volume may be extracted.

20 Examples of commercially available insulin suspension formulations include, for example, NPH, PZI, and Ultralente. A small proportion of the solid matter in a microcrystalline suspension formulation may be amorphous. Preferably, the proportion of amorphous material is less than 10%, and most 25 preferably, less than 1% of the solid matter in a microcrystalline suspension. Likewise, a small proportion of the solid matter in an amorphous precipitate suspension may be microcrystalline.

The term "aqueous solvent" refers to a liquid 30 solvent that contains water. An aqueous solvent system may be comprised solely of water, may be comprised of water plus one or more miscible solvents, and may contain solutes. The more commonly-used miscible solvents are the short-chain

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organic alcohols, such as, methanol, ethanol, propanol, short-chain ketones, such as acetone, and polyalcohols, such as glycerol.

An "isotonicity agent" is a compound that is physiologically tolerated and imparts a suitable tonicity to a formulation to prevent the net flow of water across cell membranes that are in contact with an administered formulation. Glycerol, which is also known as glycerin, is commonly used as an isotonicity agent. Other isotonicity agents include salts, e.g., sodium chloride, and monosaccharides, e.g., dextrose and lactose.

The term "preservative" refers to a compound added to a pharmaceutical formulation to act as an anti-microbial agent. A parenteral formulation must meet guidelines for preservative effectiveness to be a commercially viable multi-use product. Among preservatives known in the art as being effective and acceptable in certain formulations are benzalkonium chloride, benzethonium, chlorhexidine, phenol, m-cresol, benzyl alcohol, methylparaben, chlorobutanol, o-cresol, p-cresol, chlorocresol, phenylmercuric nitrate, thimerosal, benzoic acid, and various mixtures thereof. See, e.g., Wallhäuser, K.-H., *Develop. Biol. Standard*, 24:9-28 (1974) (S. Kräger, Basel).

The term "phenolic preservative" includes the compounds phenol, m-cresol, o-cresol, p-cresol, chlorocresol, methylparaben, and mixtures thereof. Certain phenolic preservatives, such as phenol and m-cresol, are known to bind to insulin-like molecules and thereby to induce conformational changes that increase either physical or chemical stability, or both [Birnbaum, D. T., et al., *Pharmaceutical. Res.* 14:25-36 (1997); Rahuel-Clermont, S., et al., *Biochemistry* 36:5837-5845 (1997)].

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The term "buffer" or "pharmaceutically acceptable buffer" refers to a compound that is known to be safe for use in insulin formulations and that has the effect of controlling the pH of the formulation at the pH desired for 5 the formulation. The pH of the aqueous suspension formulations of the present invention is from about 6.0 to about 8.0. Preferably the aqueous suspension formulations of the present invention have a pH between about 6.8 and about 7.8. Pharmaceutically acceptable buffers for 10 controlling pH at a moderately acidic pH to a moderately basic pH include such compounds as phosphate, acetate, citrate, arginine, TRIS, and histidine. "TRIS" refers to 2-amino-2-hydroxymethyl-1,3,-propanediol, and to any pharmacologically acceptable salt thereof. The free base 15 and the hydrochloride form are two common forms of TRIS. TRIS is also known in the art as trimethylol aminomethane, tromethamine, and tris(hydroxymethyl)aminomethane. Other buffers that are pharmaceutically acceptable, and that are suitable for controlling pH at the desired level are known 20 to the chemist of ordinary skill.

The term "administer" means to introduce a formulation of the present invention into the body of a patient in need thereof to treat a disease or condition.

The term "treating" refers to the management and 25 care of a patient having diabetes or hyperglycemia, or other condition for which insulin administration is indicated for the purpose of combating or alleviating symptoms and complications of those conditions. Treating includes administering a formulation of present invention to prevent 30 the onset of the symptoms or complications, alleviating the symptoms or complications, or eliminating the disease, condition, or disorder.

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The abbreviation "VMSED" stands for "volume mean spherical equivalent diameter." This term is well-known in the art of particle sizing. Volume mean spherical equivalent diameter is determined using a cascade impactor, 5 which measures the particle size as a function of the aerodynamic behavior of the particle in a high velocity airstream. The median (50%) particle size is obtained from a linear regression analysis of the cumulative distribution data.

10 The term "uni-modal particle size distribution" as used herein refers to a distribution of particle sizes having a single region of abundant particle sizes accompanied, for the most part, by progressively fewer particles as the particle size increases or decreases from 15 the abundant particle size region.

The term "seed crystals" is well known to one of ordinary skill in the art. It refers to a preparation of insulin-related protein crystals involving lyophilization as described in Schlichtkrull II and modifications thereof.

20 The term "Ultralente insulin" means pharmaceutical compositions containing insulin, insulin analog or derivatized insulin or derivatized insulin analog crystals prepared in acetate buffer in substantial accordance with the teaching of Hallas-Møller et.al, Science 116, 394-398 25 (1952).

The term "U" refers to the standard international unit of insulin activity.

The term "Ultralente-like crystals" refers to the crystals of insulin-related proteins described in 30 Schlichtkrull I, II and III (pages 51-52) and in Hoffmann without limitations on the VMSED, particle size distribution or the absolute morphology of the crystals.

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The crystals of the present invention have rhombohedral morphology or an irregular morphology, preferably a rhombohedral morphology.

5 The most preferred protein component in the crystals is insulin.

A preferred group of insulin analogs for preparing derivatized insulin analogs used to form crystals consists of deletion analogs and pI-shifted analogs. Deletion analogs are more preferred.

10 Another preferred group of insulin analogs for use in the crystals of the present invention consists of the monomeric insulin analogs. Particularly preferred are those monomeric insulin analogs wherein the amino acid residue at position B28 is Asp, Lys, Leu, Val, or Ala, the amino acid 15 residue at position B29 is Lys or Pro, the amino acid residue at position B10 is His, the amino acid residue at position B1 is Phe, Asp or deleted alone or in combination with a deletion of the residue at position B2, the amino acid residue at position B30 is Thr, Ala, Ser, or deleted, 20 and the amino acid residue at position B9 is Ser or Asp; provided that either position B28 or B29 is Lys.

Another preferred group of insulin analogs for use in the present invention consists of those wherein the isoelectric point of the insulin analog is between about 7.0 25 and about 8.0. These analogs are referred to as "pI-shifted insulin analogs." Examples of pI-shifted insulin analogs include, for example, ArgB31,ArgB32-human insulin, GlyA21,ArgB31,ArgB32-human insulin, ArgA0,ArgB31,ArgB32-human insulin, and ArgA0,GlyA21,ArgB31,ArgB32-human insulin.

30 Another preferred group of insulin analogs consists of LysB28,ProB29-human insulin (B28 is Lys; B29 is Pro); AspB28-human insulin (B28 is Asp), AspB1-human insulin, ArgB31,ArgB32-human insulin, ArgA0-human insulin,

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AspB1,GluB13-human insulin, AlaB26-human insulin, GlyA21-human insulin, des(ThrB30)-human insulin, and GlyA21,ArgB31,ArgB32-human insulin.

Especially preferred insulin analogs include
5 LysB28,ProB29-human insulin, des(ThrB30)-human insulin, AspB28-human insulin, and AlaB26-human insulin. Another especially preferred insulin analog is GlyA21, ArgB31, ArgB32-human insulin [Dörschug, M., U. S. Patent No. 5,656,722, 12 August 1997]. The most preferred insulin
10 analog is LysB28,ProB29-human insulin.

The preferred derivatized proteins are acylated proteins, and the preferred acylated proteins for the microcrystals and formulations of the present invention are fatty acid-acylated insulin and fatty acid-acylated insulin
15 analogs. Fatty acid-acylated human insulin is highly preferred. Fatty acid-acylated insulin analogs are also highly preferred.

The particular group used to derivatize insulin or an insulin analog (collectively, protein) may be any
20 chemical moiety that does not significantly reduce the biological activity of the protein, is not toxic when bonded to the protein, and most importantly, reduces the aqueous solubility, raises the lipophilicity, or decreases the
25 solubility of zinc/protamine complexes of the derivatized protein.

One preferred group of acylating moieties consists of fatty acids that are straight chain saturated. This group consists of methanoic acid (C1), ethanoic acid (C2), propanoic acid (C3), n-butanoic acid (C4), n-pentanoic acid
30 (C5), n-hexanoic acid (C6), n-heptanoic acid (C7), n-octanoic acid (C8), n-nonanoic acid (C9), n-decanoic acid (C10), n-undecanoic acid (C11), n-dodecanoic acid (C12), n-tridecanoic acid (C13), n-tetradecanoic acid (C14), n-

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pentadecanoic acid (C15), n-hexadecanoic acid (C16), n-heptadecanoic acid (C17), and n-octadecanoic acid (C18). Adjectival forms are formyl (C1), acetyl (C2), propionyl (C3), butyryl (C4), pentanoyl (C5), hexanoyl (C6), heptanoyl (C7), octanoyl (C8), nonanoyl (C9), decanoyl (C10), undecanoyl (C11), dodecanoyl (C12), tridecanoyl (C13), tetradecanoyl (C14) or myristoyl, pentadecanoyl (C15), hexadecanoyl (C16) or palmitic, heptadecanoyl (C17), and octadecanoyl (C18).

10 The skilled person will appreciate that narrower preferred groups are made by combining the preferred groups of fatty acids described above.

Another preferred group of acylating moieties consists of saturated fatty acids that are branched. A 15 branched fatty acid has at least two branches. The length of a "branch" of a branched fatty acid may be described by the number of carbon atoms in the branch, beginning with the acid carbon. For example, the branched fatty acid 3-ethyl-5-methylhexanoic acid has three branches that are five, six, 20 ix carbons in length. In this case, the "longest" branch is six carbons. As another example, 2,3,4,5-tetraethyloctanoic acid has five branches that are 4, 5, 6, 7, and 8 carbons long. The "longest" branch is eight carbons. A preferred group of branched fatty acids are those having from three to 25 ten carbon atoms in the longest branch.

A representative number of such branched, saturated fatty acids will be mentioned to assure the reader's comprehension of the range of such fatty acids that may be used as acylating moieties of the proteins in the 30 present invention: 2-methyl-propioinic acid, 2-methyl-butyric acid, 3-methyl-butyric acid, 2,2-dimethyl-propionic acid, 2-methyl-pentanoic acid, 3-methyl-pentanoic acid, 4-methyl-pentanoic acid, 2,2-dimethyl-butyric acid, 2,3-

dimethyl-butyric acid, 3,3-dimethyl-butyric acid, 2-ethyl-butyric acid, 2-methyl-hexanoic acid, 5-methyl-hexanoic acid, 2,2-dimethyl-pentanoic acid, 2,4-dimethyl-pentanoic acid, 2-ethyl-3-methyl-butyric acid, 2-ethyl-pentanoic acid,
5 3-ethyl-pentanoic acid, 2,2-dimethyl-3-methyl-butyric acid, 2-methyl-heptanoic acid, 3-methyl-heptanoic acid, 4-methyl-heptanoic acid, 5-methyl-heptanoic acid, 6-methyl-heptanoic acid, 2,2-dimethyl-hexanoic acid, 2,3-dimethyl-hexanoic acid, 2,4-dimethyl-hexanoic acid, 2,5-dimethyl-
10 hexanoic acid, 3,3,-dimethyl-hexanoic acid, 3,4-dimethyl-hexanoic acid, 3,5-dimethyl-hexanoic acid, 4,4-dimethyl-hexanoic acid, 2-ethyl-hexanoic acid, 3-ethyl-hexanoic acid, 4-ethyl-hexanoic acid, 4-ethyl-hexanoic acid, 2-propyl-pentanoic acid, 2-ethyl-hexanoic acid, 3-ethyl-hexanoic acid, 4-ethyl-hexanoic acid,
15 2-(1-propyl)pentanoic acid, 2-(2-propyl)pentanoic acid, 2,2-diethyl-butyric acid, 2,3,4-trimethyl-pentanoic acid, 2-methyl-octanoic acid, 4-methyl-octanoic acid, 7-methyl-octanoic acid, 2,2-dimethyl-heptanoic acid, 2,6-dimethyl-heptanoic acid, 2-ethyl-2-methyl-hexanoic acid, 3-ethyl-5-
20 methyl-hexanoic acid, 3-(1-propyl)-hexanoic acid, 2-(2-butyl)-pentanoic acid, 2-(2-(2-methylpropyl))pentanoic acid,
2-methyl-nonanoic acid, 8-methyl-nonanoic acid, 6-ethyl-octanoic acid, 4-(1-propyl)-heptanoic acid, 5-(2-propyl)-heptanoic acid, 3-methyl-undecanoic acid, 2-pentyl-
25 heptanoic acid, 2,3,4,5,6-pentamethyl-heptanoic acid, 2,6-diethyl-octanoic acid, 2-hexyl-octanoic acid, 2,3,4,5,6,7-hexamethyl-octanoic acid, 3,3-diethyl-4,4-diethyl-hexanoic acid, 2-heptyl-nonanoic acid, 2,3,4,5-tetraethyl-octanoic acid; 2-octyl-decanoic acid, and 2-(1-propyl)-3-(1-propyl)-
30 4,5-diethyl-6-methyl-heptanoic acid.

Yet another preferred group of acylating moieties consists of cyclic alkyl acids having from 5 to 24 carbon atoms, wherein the cyclic alkyl moiety, or moieties, have 5

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to 7 carbon atoms. A representative number of such cyclic alkyl acids will be mentioned to assure the reader's comprehension of the range of such acids that may be used as acylating moieties of the proteins in the present invention:

5 cyclopentyl-formic acid, cyclohexyl-formic acid, 1-cyclopentyl-acetic acid, 2-cyclohexyl-acetic acid, 1,2-dicyclopentyl-acetic acid, and the like.

A preferred group of derivatized insulins consists of mono-acylated insulin. Mono-acylation at the ϵ -amino group is most preferred. For insulin, mono-acylation at LysB29 is preferred. Similarly, for certain insulin analogs, such as, LysB28, ProB29-human insulin analog, mono-acylation at the ϵ -amino group of LysB28 is most preferred. Mono-acylation at the α -amino group of the B-chain (B1) is also preferred. Mono-acylation at the α -amino group of the A-chain (A1) is also preferred.

Another group of acylated insulins consists of di-acylated insulin. The di-acylation may be, for example, at the ϵ -amino group of Lys and at the α -amino group of the B-chain, or may be at the ϵ -amino group of Lys and at the α -amino group of the A-chain, or may be at the α -amino group of the A-chain and at the α -amino group of the B-chain.

Another group of acylated insulins consists of tri-acylated insulin. Tri-acylated proteins are those that are acylated at the ϵ -amino group of Lys, at the α -amino group of the B-chain, and at the α -amino group of the A-chain.

Aqueous compositions containing water as the major solvent are preferred. Aqueous suspensions wherein water is the solvent are highly preferred.

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The compositions of the present invention are used to treat patients who have diabetes or hyperglycemia. The dry powder or aqueous suspension formulations of the present invention will typically provide derivatized protein at 5 concentrations of from about 1 mg/mL to about 20 mg/mL. Present formulations of insulin products are typically characterized in terms of the concentration of units of 10 insulin activity (units/mL), such as U40, U50, U100, and so on, which correspond roughly to about 1.4, 1.75, and 3.5 mg/mL preparations, respectively. The dose and the number 15 of administrations per day will be determined by a physician considering such factors as the therapeutic objectives, the nature and cause of the patient's disease, the patient's gender and weight, level of exercise, eating habits, the 20 method of administration, and other factors known to the skilled physician. In broad range, a daily dose would be in the range of from about 1 nmol/kg body weight to about 6 nmol/kg body weight (6 nmol is considered equivalent to about 1 unit of insulin activity). A dose of between about 2 and about 3 nmol/kg is typical of present insulin therapy.

The physician of ordinary skill in treating diabetes will be able to select the therapeutically most advantageous means to administer liquid suspension and dry powder formulations of the present invention. The crystals 25 of the present invention are particularly well-suited for pulmonary administration.

In the aqueous, liquid suspensions, sodium chloride at 5-9 mg/mL is preferred as an isotonicity agent. Yet more highly preferred for isotonicity is to use sodium 30 chloride at a concentration of from about 6 mg/mL to about 8 mg/mL.

Insulin or insulin analogs used to prepare derivatized proteins can be prepared by any of a variety of

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recognized peptide synthesis techniques including classical (solution) methods, solid phase methods, semi-synthetic methods, and more recent recombinant DNA methods. For example, see Chance, R. E., et al., U.S. Patent No.

5 5,514,646, 7 May 1996; EPO publication number 383,472, 7 February 1996; Brange, J. J. V., et al. EPO publication number 214,826, 18 March 1987; and Belagaje, R. M., et al., U.S. Patent No. 5,304,473, 19 April 1994, which disclose the preparation of various proinsulin and insulin analogs.

10 Generally, derivatized proteins are prepared using methods known in the art. The publications listed above to describe derivatized proteins contain suitable methods to prepare derivatized proteins. To prepare acylated proteins, the protein is reacted with an activated organic acid, such
15 as an activated fatty acid. Activated fatty acids are derivatives of commonly employed acylating agents, and include activated esters of fatty acids, fatty acid halides, activated amides of fatty acids, such as, activated azolide derivatives [Hansen, L. B., WIPO Publication No. 98/02460, 20 22 January 1998], and fatty acid anhydrides. The use of activated esters, especially N-hydroxysuccinimide esters of fatty acids, is a particularly advantageous means of acylating a free amino acid with a fatty acid. Lapidot, et al. describe the preparation of N-hydroxysuccinimide esters
25 and their use in the preparation of N-lauroyl-glycine, N-lauroyl-L-serine, and N-lauroyl-L-glutamic acid. The term "activated fatty acid ester" means a fatty acid which has been activated using general techniques known in the art [Riordan, J. F. and Vallee, B. L., *Methods in Enzymology*,
30 XXV:494-499 (1972); Lapidot, Y., et al., *J. Lipid Res.* 8:142-145 (1967)]. Hydroxybenzotriazole (HOBT), N-hydroxysuccinimide and derivatives thereof are particularly

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well known for forming activated acids for peptide synthesis.

To selectively acylate the ϵ -amino group, various protecting groups may be used to block the α -amino groups during the coupling. The selection of a suitable protecting group is known to one skilled in the art and includes *p*-methoxybenzoxycarbonyl (pmZ). Preferably, the ϵ -amino group is acylated in a one-step synthesis without the use of amino-protecting groups. A process of selective acylation at the N ϵ -amino group of Lys is disclosed and claimed by Baker, J. C., et al., U.S. Patent No. 5,646,242, 8 July 1997. A process for preparing a dry powder of an acylated protein is disclosed and claimed by Baker, J. C., et al., U.S. Patent No. 5,700,904, 23 December 1997.

The primary role of divalent metal cations such as zinc in the present invention is to facilitate formation of hexamers of the protein. Zinc facilitates the formation of hexamers of insulin and insulin analogs. Zinc likewise promotes the formation of hexamers of derivatized insulin and insulin analogs. The insulins in the Ultralente-like crystals of the present invention are also believed to exist primarily in a hexameric state.

For efficient yield of crystals, the molar ratio of zinc to total protein is bounded at the lower limit by about 0.33, that is, the approximately two zinc atoms per hexamer which are needed for efficient hexamerization. Crystals will form suitably with about 2 to about 4-6 zinc atoms present when no compound that competes with insulin for zinc binding is present. Even more zinc may be used during the process if a compound that competes with the protein for zinc binding, such as one containing citrate or phosphate, is present. Excess zinc above the minimum amount

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needed for efficient hexamerization may be desirable to more strongly drive hexamerization. Also, excess zinc above the minimum amount can be present in a formulation of the present invention, and may be desirable to improve chemical 5 and physical stability, to improve suspendability, and possibly to further extend time-action. Consequently, there is a fairly wide range of zinc:protein ratios allowable in the insoluble compositions, processes, suspension and dry-powder formulations of the present invention.

10 The zinc compound that provides zinc for the present invention may be any pharmaceutically acceptable zinc compound. The addition of zinc to insulin preparations is known in the art, as are pharmaceutically acceptable sources of zinc. Preferred zinc compounds to supply zinc 15 ions for the present invention include zinc chloride, zinc acetate, zinc citrate, zinc oxide, and zinc nitrate.

In aqueous suspension formulations of the present invention, a preservative may be present, especially if the formulation is intended to be sampled multiple times. As 20 mentioned above, a wide range of suitable preservatives are known. Preferably, the preservative is present in the suspension formulation in an amount suitable to provide an antimicrobial effect sufficient to meet pharmacopoeial requirements.

25 Suitable phenolic preservatives include, for example, phenol, m-cresol, and methylparaben. The preferred preservative is methylparaben. Preferred concentrations for the preservative are from about 2 mg to about 5 mg per milliliter of the aqueous suspension formulation. These 30 concentrations refer to the total mass of phenolic preservatives because mixtures of individual phenolic preservatives are contemplated.

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The crystals of the present invention are preferably rhombohedral shaped, single crystals that are comprised of insulin, an insulin analog, a derivatized insulin or a derivatized insulin analog, and a divalent metal cation, preferably zinc.

5 The present invention provides a variety of processes for preparing the crystals. One such process is summarized;

A "protein solution" is prepared by suspending 10 bulk zinc crystals of the insulin, insulin analog, or derivatized insulin or insulin analog in an aqueous medium containing zinc oxide. Additional zinc oxide may be added. An acidic solution such as 10% hydrochloric acid is added to solubilize the protein as the suspension is adjusted to 15 about pH 2.5 to about pH 3. This protein solution is further diluted about 10-fold with water to complete preparation of the protein solution.

A "precipitation solution" is prepared that contains about 250 mg/ml sodium chloride and about 29 mg/ml 20 of sodium acetate. The precipitation solution is adjusted to about pH 11 to about 12.5 with a solution such as 10 percent sodium hydroxide and diluted further with water.

Test combinations of the protein solution and the precipitation solution are made in the ratio of 0.625 parts 25 to 0.375 parts, respectively. The pH of these test combinations are checked. Adjustments of the precipitation solution are made by, for example, adding more 10 percent sodium hydroxide, so that the combination of the two solutions in the prescribed ratio gives the optimal pH for 30 crystallization. As an example, for human insulin crystals of the present invention, the optimal pH is about pH 5.5 to about pH 5.6.

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A "nucleating seed suspension" is prepared from human insulin using methodology based on Schlichtkrull II and procedures well known in the art. This suspension contains about 0.3 mg/ml human insulin, about 0.07 mg/ml zinc, about 0.29 mg/ml anhydrous citric acid and about 0.8 mg/ml methylparaben. The seed particles are about 1 micron in diameter. The suspension is at about pH 7.9.

For preparation of the crystallization solution, the protein solution, the precipitation solution and, 10 optionally, water are combined in an appropriately sized vessel and combined by stirring. Optionally, a crystallization solution may be prepared by combining the protein with a buffer, a salt and zinc and adjusting the solution to an optimal pH for crystallization. Then, an 15 appropriate volume of the nucleating seed suspension is added. Additional acidic zinc oxide solution may be added to adjust the total zinc concentration.

Conditions considered important to this process include the following composition in the seeded 20 crystallization solution; a total protein concentration of about 0.3 to about 30 mg/ml, preferably 1 to about 20 mg/ml; a divalent metal cation such as zinc at a concentration from 1 mole per mole of protein to about 100 moles per mole of protein, preferably from 2 to about 50 moles per mole of 25 protein and more preferably from about 4 to about 20 moles per mole of protein; a pH value from about 1 pH unit above to about 1 pH unit below the pI of the protein being crystallized, preferably a pH value from about 0.5 pH units above to about 0.5 pH units below the pI of the protein; a 30 sodium chloride concentration of about 1 to about 30 mg/ml; and a sodium acetate concentration of about 0.5 to about 15 mg/ml.

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The protein is preferably human insulin at a preferred concentration of about 1.5 mg/ml and a preferred pH of about 5.5 to 5.6.

5 The "seeded crystallization solution" is preferably stirred at ambient temperature for about 24 hours.

In a preferred embodiment, the Ultralente-like crystals of the present invention are prepared in a manner that obviates the need to separate the microcrystals from 10 the mother liquor. Thus, it is preferred that the mother liquor itself be suitable for administration to the patient, or that the mother liquor can be made suitable for administration by dilution with a suitable diluent. The term diluent will be understood to mean a solution comprised 15 of an aqueous solvent in which is dissolved various pharmaceutically acceptable excipients, including without limitation, a buffer, an isotonicity agent, zinc, and the like. For further information on the variety of techniques using conventional excipients or carriers for parenteral 20 products, please see Remington's Pharmaceutical Sciences, 17th Edition, Mack Publishing Company, Easton, PA, USA (1985).

In the broad practice of the present invention, it is contemplated that a suspension of the crystals may be 25 prepared. Examples of such pharmaceutical compositions include sterile, isotonic, aqueous suspensions of insulin, an insulin analog, a derivatized insulin, or a derivatized insulin analog, buffered with a pharmaceutically acceptable buffer and pyrogen-free.

30 The present invention also provides means of isolating the crystals by procedures well known to those of ordinary skill in the art, such as filtration or centrifugation. With such techniques, the crystals may be

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washed one or more times with solutions containing only water or solutions comprising salts, buffers and optionally other excipients. Hence, many or most of the components present during the crystallization may be eliminated by 5 these crystal isolation steps. The crystals may be further resuspended in a sterile aqueous solution with components suitable for pharmaceutical use. Such ingredients could be a pharmaceutically acceptable buffer, an isotonicity agent, pharmaceutically acceptable preservatives, divalent metal 10 cations and the like. The suspension is optimized to provide stable crystals in a pharmaceutically acceptable, stable formulation useful for treating diabetes and hyperglycemia by pulmonary delivery.

Alternatively, the present invention provides 15 further that the crystals isolated from the crystallization solution by, for example filtration or centrifugation, may be washed with appropriate solutions in preparation for ultimate drying procedures. Such drying could be performed by techniques well known to those skilled in the art, such 20 as lyophilization, spray drying, vacuum drying and the like.

An effective dose of crystals for inhalation requires inhalation of from about 0.5 µg/kg to about 200 µg/kg total protein. Preferably the dose is about 5 µg/kg to about 100 µg/kg, about 10 µg/kg to about 100 µg/kg, about 25 20 µg/kg to about 100 µg/kg, or about 30 µg/kg to about 100 µg/kg. More preferably, the dose is from about 10 µg/kg to about 60 µg/kg, 20 µg/kg to about 60 µg/kg, or 30 µg/kg to about 60 µg/kg. A therapeutically effective amount can be determined by a knowledgeable practitioner, who will take 30 into account factors including insulin protein level, the physical condition of the patient, the patient's pulmonary status, the potency and bioavailability of the proteins,

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whether the total proteins are administered together with another insulin, such as a fast-acting, or meal-time insulin, or with other therapeutic agents, or other factors known to the medical practitioner. Effective starting 5 therapy can include "titration" of the patient, that is, starting at a low dose, monitoring blood glucose levels, and increasing the dose as required to achieve desired blood glucose levels.

According to the invention, crystals are delivered 10 by inhalation to achieve advantageous slow uptake of insulin protein compared both to inhalation of soluble or amorphous insulin protein. Administration by inhalation results in pharmacokinetics comparable to subcutaneous administration of crystalline insulins.

According to the present invention, crystals are delivered by any of a variety of inhalation devices and methods known in the art for administration of insulin, or other proteins, by inhalation [Rubsamen, U.S. Patent No. 5,364,838, issued 15 November, 1994; Rubsamen, U.S. Patent 15 No. 5,672,581, issued September 30, 1997; Platz, et al., WIPO publication No. WO96/32149, published October 17, 1996; Patton, et al., WIPO publication No. WO95/24183, published 20 September 14, 1995; Johnson, et al., U.S. Patent No. 5,654,007, issued August 5, 1997; Goodman, et al., U.S. Patent No. 5,404,871, issued April 11, 1995; Rubsamen, et 25 al., U.S. Patent No. 5,672,581, issued September 30, 1997; Gonda, et al., U.S. Patent No. 5,743,250, issued April 28, 1998; Rubsamen, U.S. Patent No. 5,419,315, issued May 30, 1995; Rubsamen, et al., U.S. Patent No. 5,558,085, issued 30 September 24, 1996; Gonda, et al., WIPO publication No. WO98/33480, published August 6, 1998; Rubsamen, U.S. Patent No. 5,364,838, issued November 15, 1994; Laube, et al., U.S. Patent No. 5,320,094, issued June 14, 1994; Eljamal, et al.

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U.S. Patent No. 5,780,014, issued July 14, 1998; Backstrom,
et al., U.S. Patent NO. 5,658,878, issued August 19, 1997;
Backstrom, et al., 5,518,998, issued May 21, 1996;
Backstrom, et al., 5,506,203, issued April 9, 1996; Meezan,
5 et al., U.S. Patent No. 5,661,130, issued August 26, 1997;
Hodson, et al., U.S. Patent No. 5,655,523, issued August
12, 1997; Schultz, et al., U.S. Patent No. 5,645,051, issued
July 8, 1997; Eisele, et al., U.S. Patent No. 5,622,166,
issued April 22, 1997; Mecikalski, et al., U.S. Patent No.
10 5,577,497, November 26, 1996; Mecikalski, et al., U.S.
Patent No. 5,492,112, issued February 20, 1996; Williams, et
al., U.S. Patent No. 5,327,883, issued July 12, 1994;
Williams, U.S. Patent No. 5,277,195, issued January 11,
1994].

15 Included among the devices used to administer
crystals according to the present invention are those well-
known in the art, such as, metered dose inhalers, liquid
nebulizers, dry powder inhalers, sprayers, thermal
vaporizers, and the like, and those provided by developing
20 technology, including the AERx® pulmonary drug delivery
system being developed by Aradigm Corporation, the dry
powder formulation and delivery devices being developed by
Inhale Therapeutic Systems, Inc., and the Spiros® dry powder
inhaler system being developed by Dura Pharmaceuticals, Inc.
25 Other suitable technology includes electrohydrodynamic
aerosolizers. The inhalation device should deliver small
particles, e.g., less than about 10 microns VMSED,
preferably about 1-5 microns VMSED for good respirability,
and more preferably in the range of about 1 to about 3
30 microns VMSED, and most preferably from about 2 to about 3
microns VMSED.

 In addition, the inhalation device must be
practical, in the sense of being easy to use, small enough

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to carry conveniently, capable of providing multiple doses, and durable. Some specific examples of commercially available inhalation devices suitable for the practice of this invention are Turbohaler (Astra), Rotahaler (Glaxo),
5 Diskus (Glaxo), the Ultravent nebulizer (Mallinckrodt), the Acorn II nebulizer (Marquest Medical Products), the Ventolin metered dose inhaler (Glaxo), the Spinhaler powder inhaler (Fisons), or the like. Insulin-like proteins can be advantageously delivered by a dry powder inhaler or a
10 sprayer. There are several desirable features of a dry powder inhalation device for administering crystals. For example, delivery by such inhalation devices is advantageously reliable, reproducible, and accurate.

As those skilled in the art will recognize, the
15 nature and quantity of the pharmaceutical composition, and the duration of administration of a single dose depend on the type of inhalation device employed. For some aerosol delivery systems, such as nebulizers, the frequency of administration and length of time for which the system is
20 activated will depend mainly on the concentration of crystals in the aerosol. For example, shorter periods of administration can be used at higher concentrations of crystals in the nebulizer solution. Devices such as metered dose inhalers can produce higher aerosol
25 concentrations, and can be operated shorter periods to deliver the desired amount of crystals. Devices such as dry powder inhalers deliver active agent until a given charge of agent is expelled from the device. In this type of inhaler, the amount of crystals in a given quantity of the powder
30 determines the dose delivered in a single administration.

The particle size of the crystals delivered by the inhalation device determines the extent to which the particles are conveyed into the lower airways or alveoli,

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where deposition is most advantageous because of the large surface area. Preferably, at least about 10 percent of the crystals are deposited in the lower lung, preferably about 10 percent to about 20 percent or more. It is known that
5 the maximum efficiency of pulmonary deposition for mouth-breathing humans is obtained at about 2 μm to about 3 microns VMSED. Above about 5 microns VMSED pulmonary deposition decreases substantially. Below about 1 microns VMSED pulmonary deposition decreases and it becomes
10 difficult to deliver particles with sufficient mass to be therapeutically effective. Preferably, the crystals have a particle size less than about 10 microns, preferably in the range of about 1 micron to about 5 microns VMSED, and more preferably in the range of about 1 to about 3 microns VMSED,
15 and most preferably from about 2 to about 3 microns VMSED.

Dry powder generation typically employs a method such as a scraper blade or an air blast to generate particles from a solid formulation of insulin-related protein. The particles are generally generated in a
20 container and then transported into the lung of a patient via a carrier air stream. Typically, in current dry powder inhalers, the force for breaking up the solid and air flow is provided solely by the patient's inhalation. One suitable dry powder inhaler is the Turbohaler manufactured
25 by Astra. Administration by dry powder inhaler is a preferred method for crystals.

Inhalation delivery of the crystals of the present invention can be accomplished using inhaler devices such as, but not limited to, jet nebulizers, dry powder inhalers,
30 ultrasonic nebulizers, piston pump, or piezoelectric nebulizers. The liquid solutions for the nebulizers might also contain agents such as, but not limited to, buffering agents, preservatives, surfactants. Dry powder formulations

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might include spray dried powders from solutions of sugars or polyols such as, but not limited to sucrose, lactose, dextrose, mannitol, trehalose, starch, as well as buffering agents.

5 Formulations of crystals for administration from a dry powder inhaler typically include a finely divided dry powder of the crystals, preferably produced without resort to milling or other mechanical operations. The powder can also include an insulin analog to provide relatively rapid
10 onset, short duration of action, a bulking agent, buffer, carrier, excipient, another additive, or the like. Additives can be included in a dry powder formulation of crystals, for example, to dilute the powder as required for delivery from the particular powder inhaler, to facilitate
15 processing of the formulation, to provide advantageous powder properties to the formulation, to facilitate dispersion of the powder from the inhalation device, to stabilize the formulation (e.g., antioxidants or buffers), to provide taste to the formulation, or the like.

20 Advantageously, the additive does not adversely affect the patient's airways. The crystals can be mixed with an additive so that the solid formulation includes crystal particles mixed with or coated on particles of the additive. Typical additives include mono-, di-, and
25 polysaccharides; sugar alcohols and other polyols, such as, for example, lactose, glucose, raffinose, melezitose, lactitol, maltitol, trehalose, sucrose, mannitol, starch, or combinations thereof; surfactants, such as sorbitols, phosphatidyl cholines such as dipalmitoyl choline, or
30 lecithin; or the like. Typically an additive, such as a bulking agent, is present in an amount effective for a purpose described above, often at about 50% to about 90% by weight of the formulation. Additional agents known in the

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art for formulation of a protein can also be included in the formulation. See, for example, Japanese Patent No. J04041421, published February 12, 1992 (Taisho Pharmaceutical).

5 Advantageously for administration as a dry powder, the crystals have a volume mean spherical equivalent diameter of less than about 7 microns, preferably about 1 to about 5 microns, and more preferably in the range of about 1 to about 3 microns VMSED, and most preferably, from about 2
10 to about 3 microns VMSED. The preferred particle size is effective for efficient delivery to and deposition in the alveoli of the patient's lung. Preferably, the dry powder is largely composed of particles produced so that a majority of the particles have a size in the desired range.

15 A spray including crystals can be produced by forcing a suspension of crystals through a nozzle under pressure. The nozzle size and configuration, the applied pressure, and the liquid feed rate can be chosen to achieve the desired output and particle size. An electrospray can
20 be produced by an electric field in connection with a capillary or nozzle feed. Advantageously, particles delivered by a sprayer have a particle size less than about 10 microns, preferably in the range of about 1 micron to about 5 microns VMSED, and more preferably in the range of
25 about 1 to about 3 microns VMSED, and most preferably from about 2 to about 3 microns VMSED. Administration as a spray is a preferred method for crystals.

In another embodiment of the present invention,
30 the Ultralente-like crystals are incorporated into large, porous particles suitable for administration into the deep lung by inhalation ([J. Appl. Physiol. 85:379-385 (1998); Science 276:1868-1871 (1997)]. Dry powder compositions comprising Ultralente-like crystals encapsulated in a co-

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polymer such as poly-lactic-co-glycolic acid are prepared by processes known to one of skill in the art.

Formulations of crystals suitable for use with a sprayer typically include crystals in an aqueous solution at 5 a concentration of about 1 mg to about 20 mg of total protein per mL of solution. The formulation can include agents such as an excipient, a buffer, an isotonicity agent, a preservative, a surfactant, and zinc. The formulation can also include an excipient or agent stabilization of the 10 protein, such as a buffer, a reducing agent, a bulk protein, or a carbohydrate. Bulk proteins useful in formulating crystals include albumin or the like. Typical carbohydrates useful in spray formulations include sucrose, mannitol, lactose, trehalose, glucose, or the like. The spray 15 formulation can also include a surfactant, which can reduce or prevent surface-induced aggregation of the crystals caused by atomization of the solution in forming an aerosol. Various conventional surfactants can be employed, such as polyoxyethylene fatty acid esters and alcohols, and 20 polyoxyethylene sorbitol fatty acid esters. Amounts will generally range between 0.001% and 4% by weight of the formulation. Especially preferred surfactants for purposes of this invention are polyoxyethylene sorbitan monooleate, polysorbate 80, polysorbate 20, or the like. Additional 25 agents known in the art for formulation of a protein also be included in the formulation.

Crystals can be administered by a nebulizer, such as jet nebulizer or an ultrasonic nebulizer. Typically, in a jet nebulizer, a compressed air source is used to create a 30 high-velocity air jet through an orifice. As the gas expands beyond the nozzle, a low-pressure region is created, which draws a suspension of crystals through a capillary tube connected to a liquid reservoir. The suspension

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streaming from the capillary tube is sheared into unstable filaments and droplets as it exits the tube, creating an aerosol. A range of configurations, flow rates, and baffle types can be employed to achieve the desired performance

5 characteristics from a given jet nebulizer. In an ultrasonic nebulizer, high-frequency electrical energy is used to create vibrational, mechanical energy, typically by employing a piezoelectric transducer. This energy is transmitted to the suspension of crystals either directly

10 or through a coupling fluid, creating an aerosol.

Advantageously, particles containing crystals delivered by a nebulizer have a particle size less than about 7 microns, preferably in the range of about 1 micron to about 5 microns VMSED and more preferably in the range of about 1 to about 3

15 microns VMSED, and most preferably from about 2 to about 3 microns VMSED.

Formulations of crystals suitable for use with a nebulizer, either jet or ultrasonic, typically include crystals in an aqueous solution at a concentration of about

20 1 mg to about 20 mg of total protein per mL of solution. The formulation can include agents such as an excipient, a buffer, an isotonicity agent, a preservative, a surfactant, and, preferably, zinc. The formulation can also include an excipient or agent for stabilization of the protein, such as

25 a buffer, a reducing agent, a bulk protein, or a carbohydrate. Bulk proteins useful in formulating include albumin, or the like. Typical carbohydrates useful in formulating include sucrose, mannitol, lactose, trehalose, glucose, or the like. The formulation can also include a

30 surfactant, which can reduce or prevent surface-induced aggregation of the insulin-related protein caused by atomization of the solution in forming an aerosol. Various conventional surfactants can be employed, such as

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polyoxyethylene fatty acid esters and alcohols, and polyoxyethylene sorbital fatty acid esters. Amounts will generally range between 0.001 and 4% by weight of the formulation. Especially preferred surfactants for purposes 5 of this invention are polyoxyethylene sorbitan monooleate, polysorbate 80, polysorbate 20, or the like.

In a metered dose inhaler (MDI), a propellant, suspension of crystals, and any excipients or other additives are contained in a canister as a mixture including 10 a liquefied compressed gas. Actuation of the metering valve releases the mixture as an aerosol, preferably with a volume mean spherical equivalent diameter in the range of less than about 10 microns, preferably about 1 micron to about 5 microns, and more preferably in the range of about 1 to 15 about 3 microns VMSED, and, most preferably from about 2 to about 3 microns VMSED. The desired aerosol particle size can be obtained by employing a formulation of crystals produced by various methods known to those of skill in the art, including jet-milling, spray drying, critical point 20 condensation, or the like. Preferably, mechanical methods are avoided by controlled crystallization according to the present processes. Preferred metered dose inhalers include those manufactured by 3M or Glaxo and employing a hydrofluorocarbon propellant.

25 Formulations of crystals for use with a metered-dose inhaler device will include the crystals as a finely divided powder, in a suspension in a non-aqueous medium, for example, suspended in a propellant with the aid of a surfactant. The propellant may be any conventional material 30 employed for this purpose, such as chlorofluorocarbon, a hydrochlorofluorocarbon, a hydrofluorocarbon, or a hydrocarbon, including trichlorofluoromethane, dichlorodifluoromethane, dichlorotetrafluoroethanol and

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- 1,1,1,2-tetrafluoroethane, HFA-134a (hydrofluroalkane-134a), HFA-227 (hydrofluroalkane-227), or the like. Preferably the propellant is a hydrofluorocarbon. The surfactant can be chosen to stabilize the crystals as a suspension in the
- 5 propellant, to protect the active agent against chemical degradation, and the like. Suitable surfactants include sorbitan trioleate, soya lecithin, oleic acid, or the like. Additional agents or excipients can also be included in the formulation.
- 10 The present invention may be better understood with reference to the following examples. These examples are intended to be representative of specific embodiments of the invention, and are not intended as limiting the scope of the invention.
- 15

Example 1

Preparation of Crystals

A protein solution was prepared by suspending human insulin-zinc crystals containing about 0.44 percent zinc by weight in water containing acidified zinc oxide. The insulin concentration in the suspension is greater than 27 mg/ml. The acidified zinc oxide in the solution supplemented the zinc in the insulin crystals to give a total zinc ion concentration in the suspension of about 0.23 mg/ml. A solution of 10 percent hydrochloric acid was added to the suspension to adjust the pH to about pH 2.5 to 3.0. The crystals were dissolved upon stirring the suspension and diluted with water to provide an insulin concentration of about 27 mg/ml. The solution was further diluted 10-fold

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25

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with water to provide the complete protein solution.

A precipitation solution was prepared by adjusting an aqueous solution of about 250 mg/ml sodium chloride and about 29 mg/ml sodium acetate to pH 11.0 to 12.5 with a 10

-48-

percent sodium hydroxide solution. The solution was further diluted 10-fold with water. Test combinations of this solution with the complete protein were made in a ratio of 0.375 parts to 0.625 parts, respectively, and the resulting 5 pHs of the mixtures were determined. Adjustments to the precipitation solution were made with 10 percent sodium hydroxide solution so that the test combinations with the protein solution gave a pH of about 5.5 to 5.6. This adjustment thus provides the complete precipitation 10 solution.

A nucleating seed suspension was prepared from human insulin. The method employed was based on Schlichtkrull II and procedures well known in the art. This suspension contains about 0.3 mg/ml human insulin, about 15 0.07 mg/ml zinc, about 0.29 mg/ml anhydrous citric acid and about 0.8 mg/ml methylparaben. The suspension was at about pH 7.9. The particles contained in the nucleating seed suspension were approximately 1 micron in size.

The crystallization solution was prepared by 20 combining 0.52 parts (by volume) of the complete protein solution with 0.28 parts (by volume) of the complete precipitation solution and 0.08 parts (by volume) of water in a vessel and stirred with mechanical rotation. 0.12 parts (by volume) of the nucleating seed suspension was then 25 added. Additional acidified zinc oxide solution was added to supplement the total zinc in the crystallization solution to about 0.77 mg/ml. The human insulin concentration in the crystallization suspension was about 1.5 mg/ml. The pH of this suspension was determined to be about pH 5.5 to 5.6.

30 The mixture was stirred at ambient temperature for 24 hours. The suspension was analyzed microscopically to reveal small, individual rhombohedral-shaped crystals. Particle size determinations were performed using a Coulter

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Multisizer and Sampling Stand (Coulter, Inc., Miami, Florida). The aperture tube orifice was 50 to 100 microns. Resuspended sample aliquots of 0.2 to 1.0 milliliters were pipetted into 100 milliliters of a solution containing 5 ISOTON II (Coulter, Inc., Miami, Florida). Data were collected for 50 seconds and are reported as volume mean percent diameter ($D_{4,3}$). The volume mean spherical equivalent diameter particle size was determined to be 3.67 microns \pm 1.97 microns (standard deviation) with a uni-modal 10 distribution as shown in Fig. 1.

Example 2

Intratracheal Delivery of Crystals

The Ultralente-like crystals of insulin described 15 in Example 1 were instilled intratracheally to three male F334 rats at a dose of 3 mg/kg. Blood samples were collected at 0 (pre), 0.5, 1, 4, 8, 16, and 24 hours after dosing. These blood samples were centrifuged and the serum was collected to determine glucose concentrations. The 20 blood glucose determinations are presented in Table 1.

Table 1
Blood Glucose Values after
Intratracheal Instillation in Rats

| Hours | Mean Blood Glucose (mg/dl) | Standard Deviation |
|--------------|----------------------------|--------------------|
| 0 (pre-dose) | 159.4 | 25.0 |
| 0.5 | 79.0 | 14.0 |
| 1 | 97.5 | 37.8 |
| 4 | 87.6 | 25.5 |
| 8 | 80.6 | 15.7 |
| 16 | 146.3 | 15.8 |

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| | | |
|----|-------|------|
| 24 | 147.3 | 10.5 |
|----|-------|------|

This preparation of Ultralente-type insulin crystals instilled into rat trachea provided a sustained lowering of blood glucose and surprisingly high activity.

5 Based on previous experience, the extended duration of action of the Ultralente-like insulin crystals following lung instillation shown in this example may be related, at least in part, to less macrophage uptake of the crystals than expected. It is generally known to one of ordinary
10 skill in the art that particle ingestion by macrophages in the lung is substantially complete within a few hours. The surprising results of this example suggest Ultralente-like crystals of insulin are slow dissolving and yet remain available for slow dissolution and absorption into the
15 blood.

These data create a reasonable expectation that small, Ultralente-like crystals can be administered by inhalation to a patient in need of insulin to control blood glucose, that these crystals would slowly dissolve within
20 the lung and the insulin activity would be slowly absorbed into the patient's blood from the deposited particles.

The invention has been described with reference to various specific and preferred embodiments and techniques.
25 However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention. All publications and patent applications in this specification are indicative of the level of ordinary skill in the art to which this invention
30 pertains.

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We claim:

1. Ultralente-like crystals having a uni-modal
5 particle size distribution comprising:
 - a) insulin, an insulin analog, a derivatized insulin, or a derivatized insulin analog;
and
 - b) a divalent metal cation;
10 characterized in that the volume mean spherical equivalent diameter of the crystals is from 1 micron to 5 microns.
2. Crystals according to Claim 1, wherein the divalent metal cation is zinc.
- 15 3. Crystals according to Claim 2 wherein zinc is present at about 0.3 to about 2.0 mole per mole of insulin, insulin analog, derivatized insulin or derivatized insulin analog.
4. Crystals according to any one of Claims 1 - 3,
20 wherein the volume mean spherical equivalent diameter is from 1.5 microns to 4.5 microns.
5. Crystals according to Claim 4 wherein the volume mean spherical equivalent diameter is from 2 microns to 4 microns.
- 25 6. A process for preparing crystals according to any one of Claims 1 - 5, comprising:
 - a) preparing a crystallization solution comprising insulin, an insulin analog, a derivatized insulin or a derivatized insulin analog, a buffer, a salt and a divalent cation;

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b) combining the crystallization solution of step a) with a nucleating seed suspension; and

5 c). allowing time for the seeded crystallization solution of step b) to generate the crystals according to any one of Claims 1 - 5.

7. The process of Claim 6 wherein the nucleating seed suspension comprises insulin or a derivatized insulin.

10 8. The process of Claim 6 wherein the volume of nucleating seed suspension is equivalent to about 5 to about 25 percent of the volume of the seeded crystallization solution.

15 9. The process of Claim 8 wherein the volume of nucleating seed suspension is equivalent to about 8 to about 20 percent of the volume of the seeded crystallization solution.

20 10. The process of Claim 9 wherein the volume of nucleating seed suspension is equivalent to about 10 to about 15 percent of the volume of the seeded crystallization solution.

25 11. The process of Claim 6 wherein the seeded crystallization solution has a protein concentration of about 0.5 to about 20 mg/ml.

12. The process of Claim 11 wherein the seeded crystallization solution has a protein concentration of about 1 to about 10 mg/ml.

30 13. The process of Claim 12 wherein the seeded crystallization solution has a protein concentration of about 2 to about 4 mg/ml.

14. The process of Claim 6 wherein the divalent metal cation is zinc.

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15. The process of Claim 6 wherein the crystallization proceeds for 1 to about 48 hours.

16. The process of Claim 15 wherein the crystallization process proceeds for 2 to about 30 hours.

5 17. The process of Claim 16 wherein the crystallization process proceeds for 3 to about 25 hours.

18. The process of Claim 6 wherein the buffer is sodium acetate and the salt is sodium chloride.

10 19. The process of Claim 8 wherein the crystallization solution further comprises citrate.

20. A pharmaceutical composition for administration by inhalation by mouth comprising the crystals according to of any one of Claims 1 - 5.

15 21. The pharmaceutical composition of Claim 20 further comprising a carrier, an additive, an excipient, or an aqueous solvent.

22. The pharmaceutical composition of Claim 21 wherein the crystals are in the form of a dry powder.

20 23. The pharmaceutical composition of Claim 21 further comprising a non-crystalline form of insulin, an insulin analog, derivatized insulin or derivatized insulin analog.

25 24. Use of the crystals according to of any one of Claims 1 - 5 to prepare a medicament for the treatment of diabetes or hyperglycemia by mouth.

30 25. A method of using the crystals according to any one of the Claims 1 - 5 to treat diabetes or hyperglycemia using a device to administer the crystals by inhalation via the mouth to a patient in need of such treatment.

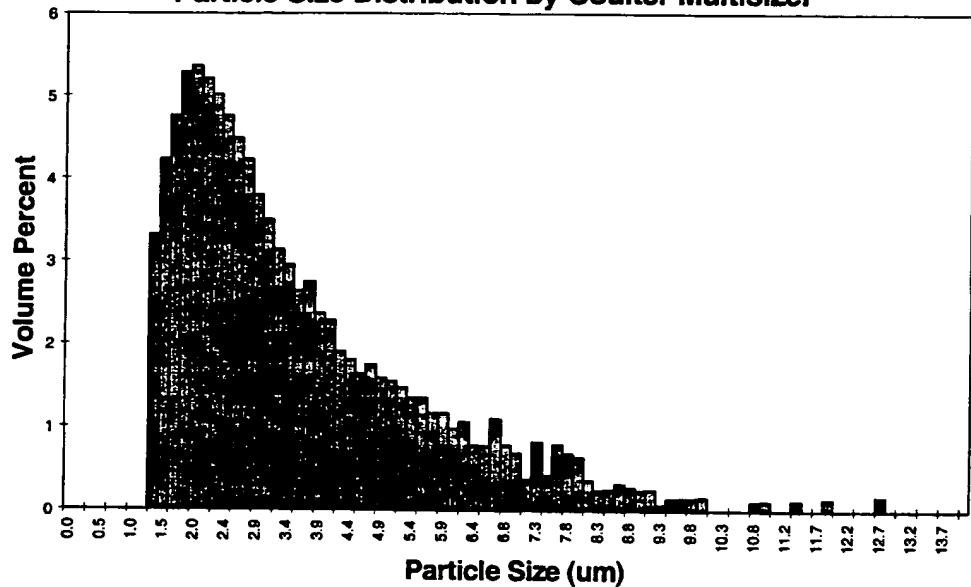
26. A method of treating diabetes comprising administering the pharmaceutical composition according to

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any one of the Claims 20 - 23 to a patient in need thereof to regulate blood glucose levels in the patient.

27. The method of treating diabetes according to Claim 26 wherein the pharmaceutical composition is
5 administered once a day to the patient.

Figure 1
Particle Size Distribution by Coulter Multisizer



5

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Sheet 1 of 1

INTERNATIONAL SEARCH REPORT

Internatinal Application No
PCT/US 00/15026

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K14/62 A61K38/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|---|-----------------------|
| X | WO 98 42367 A (HANSEN PHILIP ;JENSEN STEEN (DK); NOVONORDISK AS (DK)) 1 October 1998 (1998-10-01) page 3, line 19 - line 24 page 3, line 27 - line 28 page 4, line 5 - line 29 page 8, line 4 - line 7; claims | 1-27 |
| X | WO 98 42368 A (HANSEN PHILIP ;JENSEN STEEN (DK); NOVONORDISK AS (DK)) 1 October 1998 (1998-10-01) page 3, line 29 -page 4, line 26 page 5, line 15 - line 33; claims | 1-27 |

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

20 September 2000

04/10/2000

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Fuhr, C

INTERNATIONAL SEARCH REPORT

| | |
|-----------------|-------------------|
| Inten: | na Application No |
| PCT/US 00/15026 | |

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|---|-----------------------|
| P,X | <p>J.P. RICHARDS ET AL.: "Preparation of Microcrystalline Suspension Formulation of LysB28ProB29-Human Insulin with Ultralente Properties" <i>JOURNAL OF PHARMACEUTICAL SCIENCES</i>, 'Online! vol. 88, no. 9, 8 July 1999 (1999-07-08), pages 861-867, XP002147977 page 862, left-hand column, paragraph 2 page 863, right-hand column, paragraph 2 page 866, left-hand column, paragraph 2 -page 867, left-hand column, paragraph 1</p> <hr/> | 1-3, 6-19,26 |
| X | <p>J.L. WHITTINGHAM ET AL.: "Crystal Structure of a Prolonged-Acting Insulin with albumin-Binding Properties " <i>BIOCHEMISTRY</i>, vol. 36, 11 March 1997 (1997-03-11), pages 2826-2831, XP002147629 EASTON, PA US page 2827, left-hand column, paragraph 2 page 2830, left-hand column, paragraph 2 -page 2831, left-hand column, last paragraph</p> <hr/> | 1-3 |
| P,X | <p>EP 0 951 910 A (LILLY CO ELI) 27 October 1999 (1999-10-27) paragraph '0013! - paragraph '0014! paragraph '0018! paragraph '0029! - paragraph '0032! paragraph '0041!; claims</p> <hr/> | 1-27 |

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1-27 relate to insulin crystals defined (*inter alia*) by reference to the following parameter(s): "volume mean spherical equivalent diameter is from 1 micron to 5 micron"

The use of these parameters in the present context is considered to lead to a lack of clarity within the meaning of Article 6 PCT. It is impossible to compare the parameters the applicant has chosen to employ with what is set out in the prior art. The lack of clarity is such as to render a meaningful complete search impossible. Consequently, the search has been restricted to insulin-zinc crystals as such.

Present claims 1-27 relate to a product and composition defined by reference to a desirable characteristic or property, namely being "ultralente-like".

The claims cover all products and compositions having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such products and compositions. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the product and compositions by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to insulin-zinc crystals as such and in particular to those crystals which can be administered pulmonary.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

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|--------|-------------------|
| Intern | al Application No |
| | PCT/US 00/15026 |

| Patent document cited in search report | Publication date | Patent family member(s) | | | Publication date |
|--|------------------|-------------------------|--|--|------------------|
| WO 9842367 | A 01-10-1998 | AU 6611898 A | | | 20-10-1998 |
| | | EP 0969860 A | | | 12-01-2000 |
| | | US 6043214 A | | | 28-03-2000 |
| WO 9842368 | A 01-10-1998 | AU 6611998 A | | | 20-10-1998 |
| | | EP 0971729 A | | | 19-01-2000 |
| | | US 5898028 A | | | 27-04-1999 |
| EP 0951910 | A 27-10-1999 | AU 2107399 A | | | 26-07-1999 |
| | | AU 2312299 A | | | 26-07-1999 |
| | | EP 0951911 A | | | 27-10-1999 |
| | | NO 20003050 A | | | 14-06-2000 |
| | | NO 20003051 A | | | 14-06-2000 |
| | | WO 9934815 A | | | 15-07-1999 |
| | | WO 9934816 A | | | 15-07-1999 |